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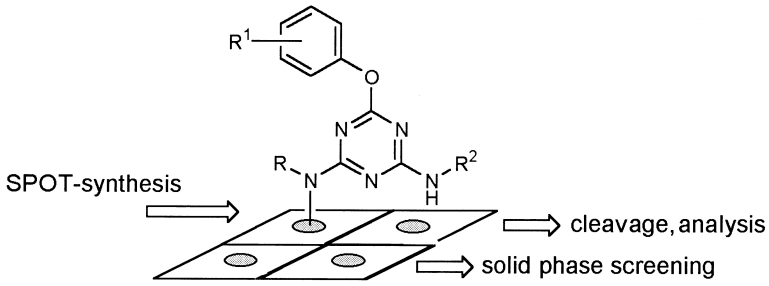

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Spatially Addressed Synthesis of Amino- and Amino-Oxy-Substituted 1,3,5-Triazine Arrays on Polymeric Membranes

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Effective spatially addressed parallel assembly of trisamino- and amino-oxy-1,3,5-triazines was achieved by applying the SPOT-synthesis technique on cellulose and polypropylene membranes. In addition to developing a suitable linker strategy and employing amines and phenolate ions as building blocks, a highly effective microwave-assisted nucleophilic substitution procedure at membrane-bound monochlorotriazines was developed. The 1,3,5-triazines obtained could be cleaved in parallel from the solid support by TFA vapor to give compounds adsorbed on the membrane surface in a conserved spatially addressed format for analysis and screening. The reaction conditions developed were employed for the synthesis of 8000 cellulose-bound 1,3,5-triazines which were probed in parallel for binding to the anti-transforming growth factor-α monoclonal antibody Tab2 in order to identify epitope mimics.

Introduction

One of the major efforts in combinatorial chemistry is focused on the construction of small organic compound libraries based on heterocyclic systems.[1] 1,3,5-Triazines are of special interest due to their synthetic accessibility and their potential therapeutic value for the treatment of, e.g., cancer[2] and depression.[3] Such compounds are synthetically available from cyanuric chloride by taking advantage of the temperature-dependent successive displacement of the chlorine atoms by different nucleophiles.[4] Earlier reports on the synthesis of combinatorial libraries derived from cyanuric chloride used the "one bead one compound" solid phase strategy to yield compound mixtures[5] or the solution phase procedures involving a high level of instrumentation and relatively harsh conditions or long reaction times.[6,7]

In contrast, the present study describes the development of a SPOT-synthesis protocol which allows the rapid generation of highly diverse spatially addressed single compounds under mild conditions. Although SPOT-synthesis[8] has been described to be ideally suited for the efficient and flexible synthesis and screening of peptides directly on cellulose membranes, assembly of small organic molecules has not been reported. To develop a reliable SPOT-synthesis protocol for efficient synthesis of small organic molecules such as 1,3,5-triazines on continuous surfaces the following prerequisites had to be fulfilled: (i) application of suitable planar polymeric supports (with high stability at increased temperatures and under reductive conditions), (ii) introduction of an orthogonal ester-free linker system cleavable under dry conditions, and (iii) development of a robust synthetic pathway (Figure 1).

Results and Discussion

Initially, in order to examine the effect of membrane composition on the chemical synthesis two different membranes were tested. In addition to cellulose, which was earlier demonstrated to be well suited for the synthesis of peptide libraries and heterogeneous screening assays,[9] two differently modified polypropylene (pp) membranes were developed which not only showed an increased chemical and mechanical stability but also lacked the excess functional groups of cellulose which might interfere in various synthetic operations. For stability, amino-functionalized membranes would be most appropriate to avoid cleavage by the strong nucleophiles used during the synthesis. Therefore, cellulose was modified with epibromohydrin and quenched with 4,7,10-trioxa-1,13-tridecanediamine[10] to yield an amino-functionalized membrane with an adjusted loading between 0.1 and 1.5 μmol/cm^2. Pp-membranes were first functionalized by photoinduced copolymerization with benzophenone (Bp) as radical initiator and acrylic acid or acrylic acid methyl ester as monomers to give membranes **1** and **2** of Scheme 1, respectively.[11] The carboxylic acid or methyl ester functionalities were then transformed into the corresponding amide-linked amines (membrane **3**) according to Scheme 1.

To activate the membrane-bound carboxyl groups, several procedures were tested (Table 1). The amount of amino functions generated was determined by reaction with activated Fmoc-β-Ala-OH, followed by Fmoc deprotection with 20% piperidine in *N*,*N*-dimethylformamide (DMF) for 30 min and measurement of the UV absorbance of the released dibenzofulvene/piperidine adduct ($\epsilon_{301} = 7800$).

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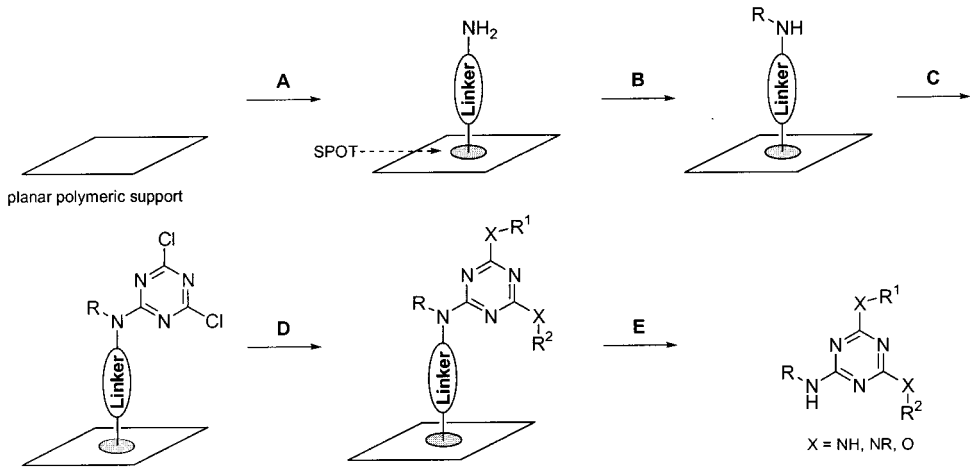


Figure 1. General synthesis strategy of 1,3,5-triazines on planar surfaces: functionalization of a suitable planar polymeric support and linker synthesis/attachment (A); introduction of the first building block (B); attachment of cyanuric chloride (C); stepwise substitution of the chlorine atoms (D); cleavage from continuous surfaces (E).

Scheme 1

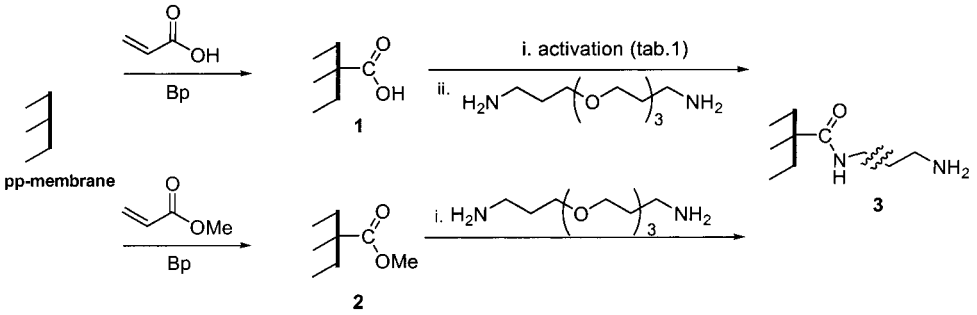


Table 1. Activation of Pp-Membrane-Bound Carboxyl Groups to Achieve High Amino Functionalization (Membrane Loading) after Amidation[a]

activation reagent	concentration	reaction time [min]	membrane loading [nmol/cm^2][a]
DIC/HOBt	1 M in NMP	30	43 ± 5
DIC/PfpOH	1 M in NMP	60	107 ± 5
$SOCl_2$	2 M in DCM	60	32 ± 6
$SOCl_2$	4 M in DCM	120	46 ± 5
$(COCl)_2$	2 M in DCM	120	193 ± 5
PCl_3	2 M in DCM	60	108 ± 6
PCl_5	2 M in DCM	60	296 ± 8
PCl_5	2 M in DCM	120	521 ± 6

[a] See Scheme 1. [b] Mean value obtained from three measurements.

Activation via PCl_5 through the intermediate acid chloride at room temperature was shown to give the best results according to membrane loading (Table 1).

Alternatively, amino functionalized pp-membranes were obtained by reaction of a methyl ester-modified pp-membrane **2** with 4,7,10-trioxa-1,13-tridecanediamine.[11] This procedure was shown to give lower overall yields following reaction for 48 h at 80 °C (200−300 nmol/cm^2 of amino functions) in comparison with the acid chloride technique. However, increasing reaction times (>72 h) and temperature (100 °C) resulted in membrane loadings (500−800 nmol/cm^2) similar to those obtained via the acid chloride procedure. To generate cleavable compound libraries for solution phase screening and quality control, an appropriate linker system first had to be established (Figure 1, A). Initially, the Rink linker[12] was attached via its pentafluorophenylester in *N*-methyl-2-pyrrolidinone (NMP) to amino-functionalized cellulose and pp-membrane **3** to give **4** and **5** of Figure 2, respectively. To synthesize structures that are not accessible via the Rink linker (see below), a carbamate-type linker was synthesized directly on the chemically more stable pp-membrane (Scheme 2). Amino membrane **3** was acylated with bromoacetyl bromide in dichloromethane (DCM) and subsequently reacted with the cesium salt of *p*-hydroxybenzaldehyde (*p*-OBA). The aldehyde functionality of **6** (Scheme 2) was reduced to the corresponding benzylic alcohol by sodium borohydride, and carbonate **7** was formed by reaction with 4-nitrophenyl chloroformate in the presence of *N*-ethylmorpholine (NEM) as base.

The *p*-nitrophenyl (Pnp) activated membrane **7** enabled attachment of bifunctional building blocks such as diamines,[13] thus enhancing the diversity of accessible compounds (Figure 2). The assembled amino functions (deprotected **4**, **5** or diamino-derivatized **7**) were subsequently modified by immersing the membranes in a 4 M solution of cyanuric chloride in DCM for 15 min to give **8a/b** (Figure 2). For further increases in diversity, membranes **4**, **5** or amino-derivatized **7** could also be used for stepwise synthesis of oligomeric structures such as peptides or peptoids[10] (shown in Figure 1, B) prior to the attachment of cyanuric chloride to yield **9** and **10**, respectively (Figure 2).

The second chlorine atom of the membrane-bound dichloro-1,3,5-triazine was selectively substituted at room temperature by pipetting 1 or 2 μL of 2−5 M solutions of primary or

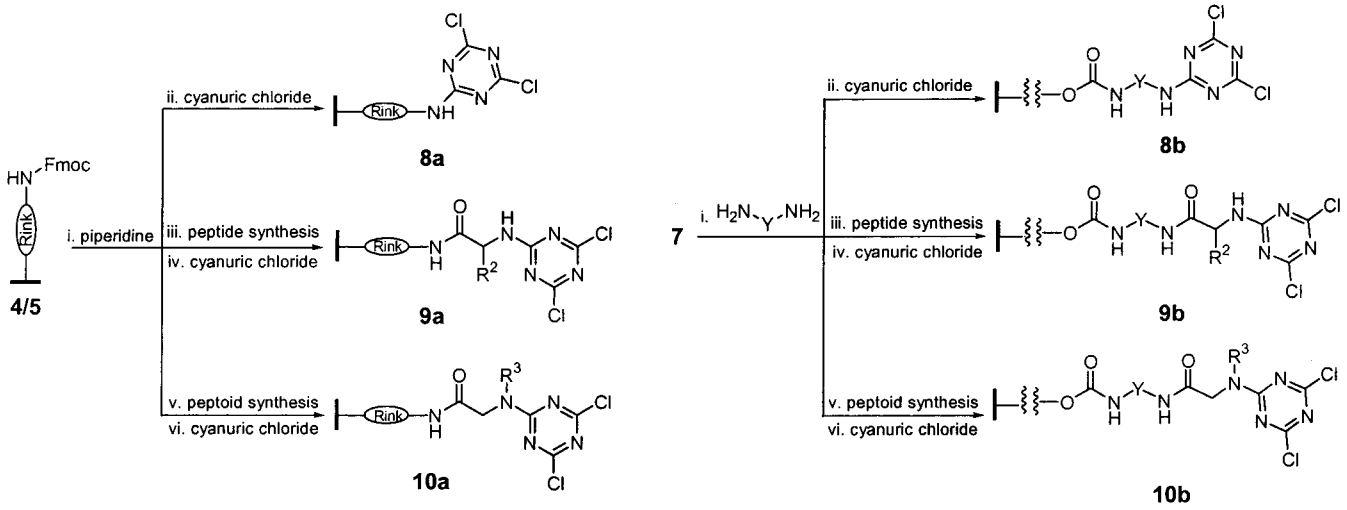


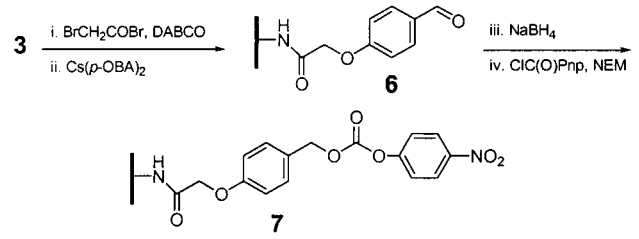
Figure 2. Modular strategy for the generation of membrane-bound dichloro-1,3,5-triazines using cellulose and pp-membranes as solid support combined with two linker systems (Rink and carbamate-type) followed by peptide or peptoid synthesis or attachment of diamines for diversity enhancement (Y = ring or chain).

Table 2. Reaction of Dichloro-1,3,5-triazine Linked to a Rink Linker Modified Membrane with Selected Amines

amine	membrane[a]	conversion[b] [%]	purity [%]
3-chloro-4-fluoroaniline	c	benzylamine/95	90
3-chloro-4-fluoroaniline	pp	benzylamine/95	95
α-naphthylamine	pp	benzylamine/45	34
α-naphthylamine + 0.1 equiv TMSCl	pp	benzylamine/75	65
1-aminoadamantane	pp	piperidine/10	10
1-aminoadamantane + 0.1 equiv TMSCl	pp	piperidine/15	10
propargylamine	c	piperidine/95	90
propargylamine	pp	piperidine/95	90
hydroxylamine	c	piperidine/95	87
hydroxylamine	pp	piperidine/95	90

[a] c = cellulose; pp = pp-membrane. [b] Nonreacted chlorines were quenched with appropriate amine at elevated temperature.[14]

Scheme 2



secondary amines in NMP onto the pp-membranes or cellulose. Droplets were positioned in the center of a 1 × 1 cm grid, which was drawn on the cellulose or pp-membrane with a soft pencil prior to the first pipetting step. A reaction time of 30 min without additional base was sufficient for substitution, when high excesses of reactants (10−20 equiv) were used.[13] In no case was the cross-linking of two triazine moieties by means of a primary amine or even a diamine (without monoprotection) observed. Amino alcohols could also be applied directly due to chemoselective reaction of the amino function with the dichlorotriazines.[6] In contrast, certain less nucleophilic amines such as anilines or sterically hindered amines yielded significantly lower substitutions (Table 2). Addition of trimethylsilyl chloride (TMSCl) (0.1 equiv relative to the amine employed) resulted in improved yields and purities for anilines, but even in this case complete substitution could not be achieved regardless of the use of longer reaction times.[14,15]

Several alcohols or phenols (cyclohexanol, 2,2,2-trifluoroethanol, benzyl alcohol, phenol, and pentafluorophenol) as 5 M solutions in NMP were tested as nucleophiles for substitution of a single chlorine atom at pp-membrane-bound dichlorotriazines. However, only low substitution levels were obtained (~10%), even in the presence of a base such as *N*,*N*-diisopropylethylamine (DIPEA). To increase the nucleophilicity of the alcohols, 0.2−1 M solutions, depending on their solubility, of their cesium salts in dimethyl sulfoxide (DMSO) were employed. No increased yields were observed for the aliphatic and benzylic alcohols, whereas with phenols nearly quantitative substitutions were obtained. To establish the applicability of this method for both cellulose and pp membranes, a set of several phenols was tested.[13] It was found that use of the corresponding cesium salts generally gave increased yields of the aryl ethers. For several phenolate ions (e.g., 4-hydroxybenzotrifluoride, (±)-1,1′-bi-(2-naphthol), and vanillin) pp-membranes resulted in better conversions than cellulose, perhaps due to quenching of the phenolate by the large excess of hydroxy functions present in the latter case (for an example, see Figure 3a,b).

To achieve complete substitution of the last chlorine atom from the monochlorotriazines, relatively harsh conditions such as 80 °C for 5 h[6] or very long reaction times (4 days)[7] were needed. The SPOT-synthesis, configured as an open system at room temperature, is not compatible with such conditions due to the fast evaporation of reactants. Thus,

Table 3. High Temperature Conditions for the Chlorine Substitution in Monochloro-1,3,5-triazines in Solution (6-Chloro-*N*-cyclohexyl-*N*′-benzyl-1,3,5-triazine-2,4-diamine) and on Solid Phase ((4-Chloro-6-piperazine-1-yl-1,3,5-triazine-2-yl)-benzyl-amine, Immobilized through a Carbamate Linker) in NMP

phase	nucleophile	condition	conversion [%][a]
solution	tetrahydrofurfurylamine	140 °C, 24 h	50
solution	tetrahydrofurfurylamine	microwave, 3 min	>95
TentaGel resin	tetrahydrofurfurylamine	80 °C, 1 h	75
TentaGel resin	tetrahydrofurfurylamine	80 °C, 4 h	>95
TentaGel resin	tetrahydrofurfurylamine	microwave, 3 min	>95
TentaGel resin	cyclohexylamine	80 °C, 1 h	50
TentaGel resin	cyclohexylamine	microwave, 3 min	>95
TentaGel resin	dibutylamine	microwave, 3 min	>95
TentaGel resin	phenol (Cs salt)	microwave, 3 min	>90
TentaGel resin	vanillin (Cs salt)	microwave, 3 min	>90

[a] Calculated from HPLC peak area of crude reaction product.

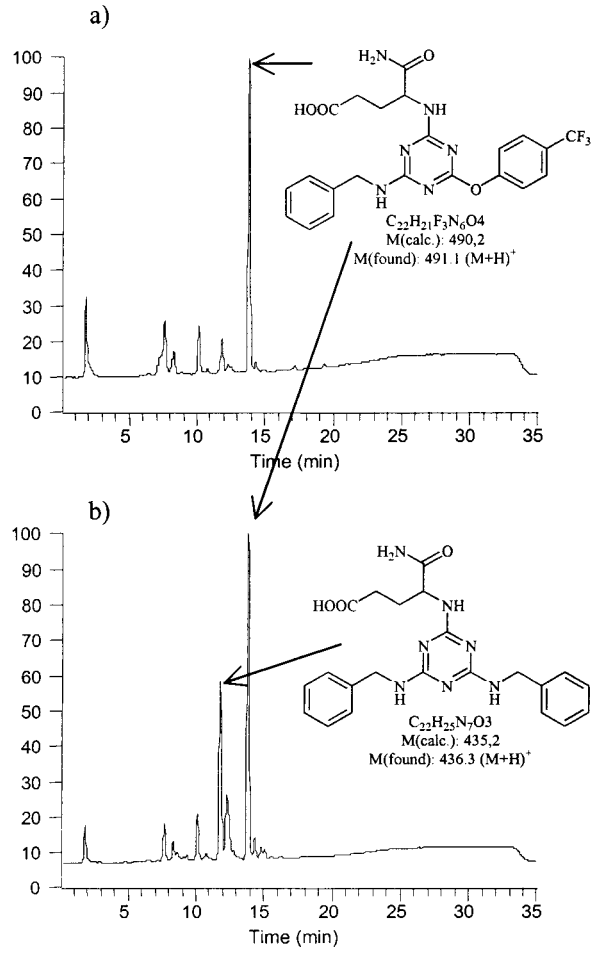


Figure 3. HPLC profiles of crude *N*-benzyl-6-(4-trifluoromethylphenoxy)-1,3,5-triazine-2,4-diamine obtained after cleavage from pp-membrane (a) and from cellulose (b). Chlorine substitution with phenolates was achieved at room temperature on the dichlorotriazines. The remaining chlorine atoms were quenched by benzylamine at elevated temperature.

conventional heater systems are not feasible for the SPOT-method on membranes since the slow energy transfer from the heater to the membrane results in an evaporation of the small volumes (1 or 2 μL) of pipetted amine solutions before the appropriate temperature is reached ($\sim$5 min). Therefore microwave irradiation, earlier shown to have a rate enhancing effect on aromatic nucleophilic substitutions in solution,[16] was tested. Initially, several model reactions were carried out in solution and on solid support to demonstrate the applicability of microwave irradiation for substitution of the chlorine atom in monochloro-1,3,5-triazines. In fact, it was found that microwave irradiation significantly increased the substitution rate (Table 3).

While examining the microwave methodology it was found that evaporation rates of solvents from the membranes were similar to those noted for conventional heater systems. However, for all membranes studied, substitutions were found to proceed within minutes ($\leq$6 min), with both amines and solutions of cesium salts of phenols. As noted earlier, aliphatic hydroxyl groups need not be protected as they do not react under the conditions used (Figure 4a). For substitution of the monochlorotriazines, a second pipetting/microwave cycle was needed for volatile amines with boiling points below 70 °C or for aniline derivatives where addition of 0.1 equiv of TMSCl was required.[13] Interestingly, applying microwave-assisted nucleophilic substitution with cesium phenolates on cellulose-bound monochlorotriazines gave significantly better yields than employing the same phenolates for substitution of a single chlorine at dichlorotriazines at room temperature. Accordingly, synthesis of mono-oxy-diamino-1,3,5-triazines is best achieved by employing the corresponding phenolate by means of microwave irradiation at membrane-bound monochlorotriazines.

The fact that the 1,3,5-triazines can be released from the solid support by trifluoroacetic acid (TFA) vapor is advantageous since the compound libraries remain adsorbed at the location of synthesis. Membrane disks (one SPOT = 0.23 cm^2), containing the adsorbed compounds, could be punched out for product identity and purity control or placed in microtiter plates for screening purposes. One SPOT yielded sufficient material (50−250 nmol, depending on adjusted loading) for multiple HPLC-MS analyses. Parallel cleavage of single membrane SPOTs with 80% TFA in DCM (v/v) for 30 min afforded similar amounts of released products, verifying completeness of the TFA vapor cleavage. Examples of HPLC traces of several crude products are displayed in Figure 4.

To evaluate the applicability of 1,3,5-triazine libraries for solid phase binding assays directly on the cellulose membrane, identification of small molecule antigen mimics binding to the anti-transforming growth factor-α (TGF-α) monoclonal antibody (mab) Tab2 was attempted. The antibody was raised against the recombinantly expressed protein antigen and binds to the linear epitope VVSHFND

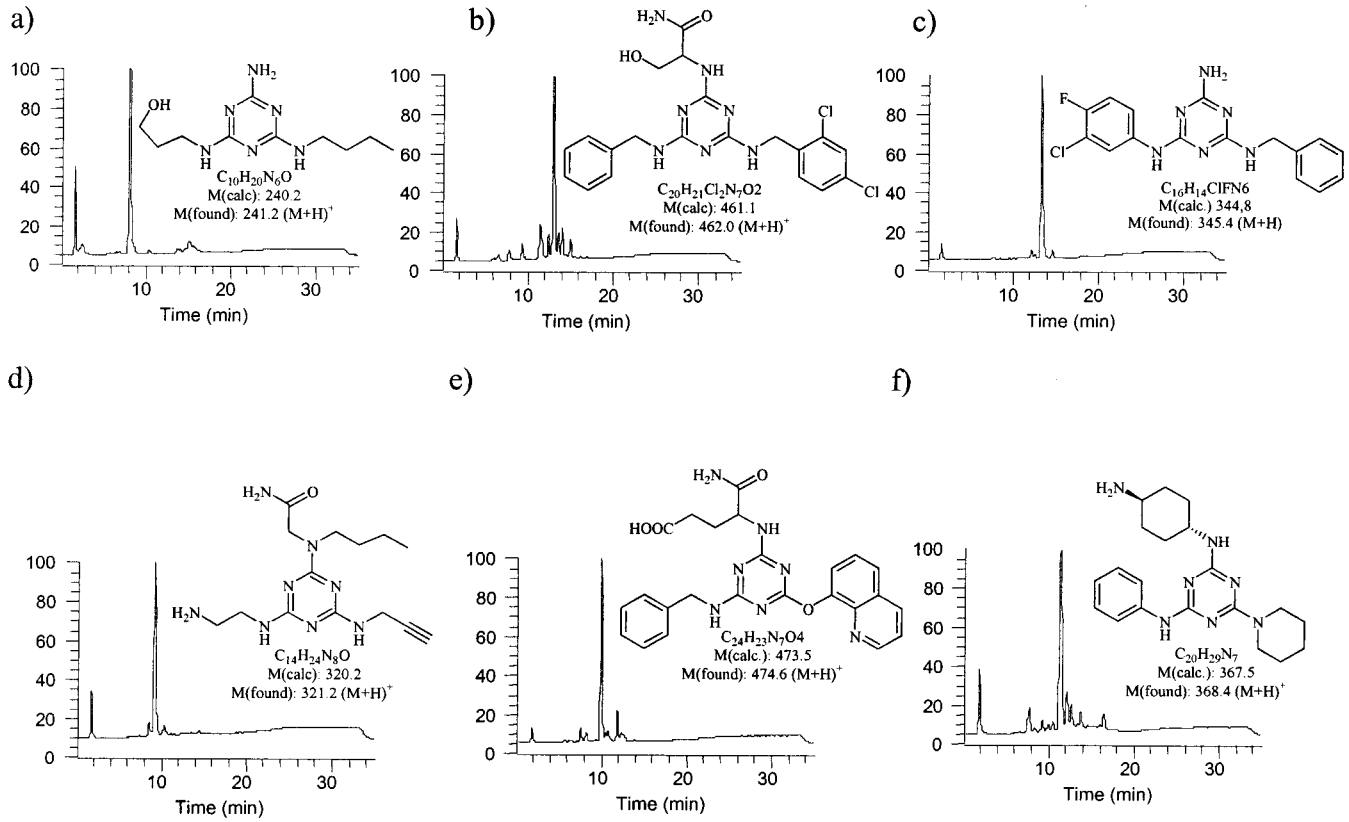
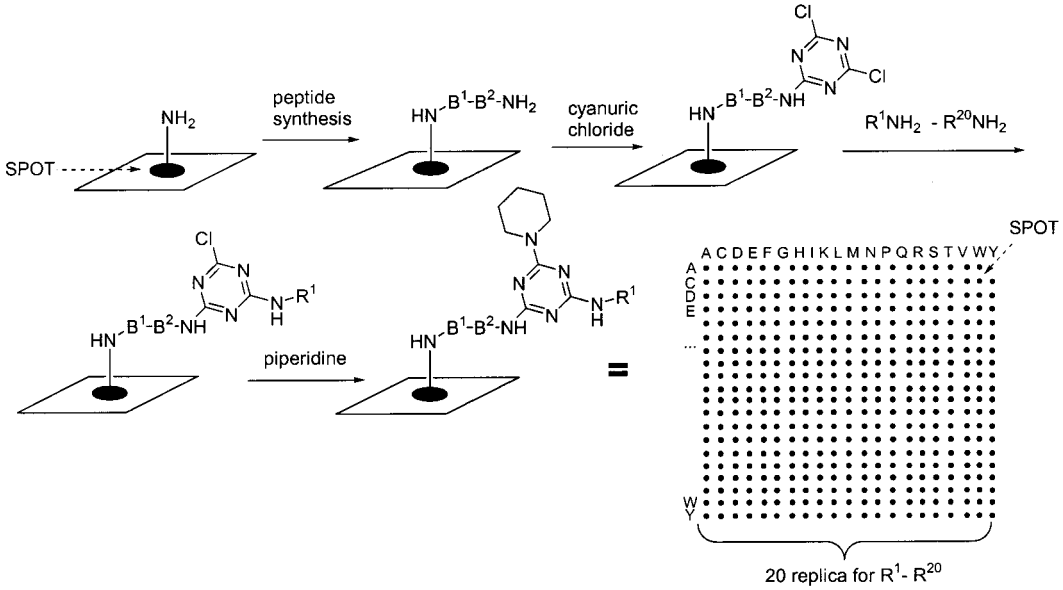


Figure 4. Elution profiles of differently substituted 1,3,5-triazines cleaved by TFA vapor (a−c from cellulose membrane; d−f from pp-membrane).

Scheme 3



at the N-terminus of TGF-α as well as to the same sequence as the 7-mer peptide.[17,9] For that purpose, 8000 different membrane-bound 1,3,5-triazines were synthesized on a 18 × 26 cm cellulose membrane with a density of 25 SPOTs per cm^2, using the optimized conditions described above. According to Scheme 3 all possible 400 dipeptides composed of the 20 proteinogenic L-amino acids (B^1 and B^2 in Scheme 3) were synthesized in 20 replica. Subsequently, cyanuric chloride was attached to each dipeptide, followed by selective "spot-wise" substitution of one of the two remaining chlorine atoms by 20 different amines (Figure 6). Finally, the chlorine atom at the membrane-bound monochlorotriazines was replaced by piperidine under microwave irradiation followed by deprotection of the side-chain functionalities of the dipeptide moiety. The library was tested for binding of the murine IgG mab Tab2 directly on the cellulose sheet followed by incubation with a peroxidase-labeled polyclonal serum against the constant regions of mouse IgG antibodies. Detection of peptide-bound Tab2/secondary antibody complexes was carried out using a chemoluminescence substrate in combination with an imager system (see Experimental Section). Several antibody binding SPOTs bearing 1,3,5-

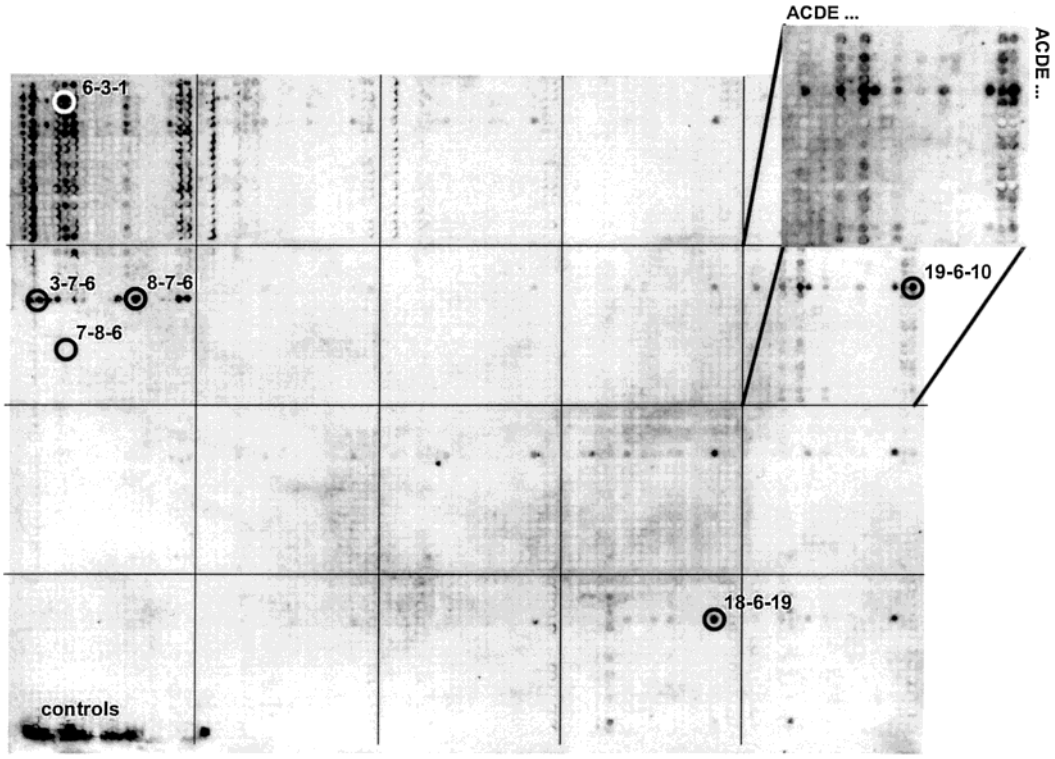
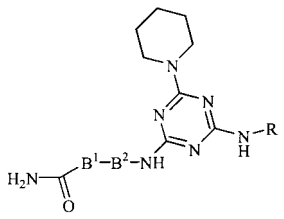


Figure 5. Membrane-bound triazines (8000) probed with the monoclonal antibody Tab2. Binding was detected by a second peroxidase-labeled antibody and a chemoluminescent substrate. Each square within the drawn grid contains 400 dipeptide-1,3,5-triazines with R^1 to R^{20} as defined by Figure 6.

Table 4. Selected Tab2-Binding Triazines in Heterogeneous and Homogeneous Screening Assays



no.	sequence B^1	sequence B^2	sequence R	intensity [BLU][a]	IC_{50} from ELISA [μmol]
18-6-19	Val	Gly	3-chlorobenzylamine	2.2×10^6	600 ± 300
19-6-10	Tyr	Gly	3-picolylamine	1.9×10^6	$>10^4$
8-7-6	Gln	His	4-(2-aminoethyl)morpholine	2.3×10^6	$>10^4$
3-7-6	Asp	His	4-(2-aminoethyl)morpholine	1.9×10^6	$>10^4$
6-3-1	Gly	Asp	aminomethylcyclohexane	1.9×10^6	400 ± 300
7-8-6	His	Gln	4-(2-aminoethyl)morpholine	1.1×10^5	$>10^4$

[a] BLU = Boehringer light units.

triazines as well as the positive control peptide VVSHFNS were observed (Figure 5). To show that the detected signals are not based on binding of the second antibody or the peroxidase itself directly to the membrane-bound compounds, a control experiment with the peroxidase-labeled anti-mouse IgG detection antibody alone was performed which showed no enzymatic activity on the membrane (data not shown). Five of the 1,3,5-triazines were selected based on the highest signal intensities and maximum diversity of the substitution pattern together with one negative control (Table 4), and these molecules were resynthesized on resin at a milligram scale and tested by competitive ELISA for their inhibitory effect on the Tab2/VVSHFND interaction. Inhibition curves were determined in duplicate and are shown in Figure 7. The inhibition constants (Table 4) were calculated as described in the Experimental Section. Two compounds (library members **18-6-19** and **6-3-1**) had a weak but significant inhibitory effect in the upper micromolar range (Table 4). However, no inhibition was measured for compounds **19-6-10**, **8-7-6**, and **3-7-6**, although Tab2 binding was detected on the cellulose membrane. This observtion can be due to three reasons: (1) The compounds do not bind to the paratope but to the constant regions of the antibody. Therefore, binding of Tab2 is observed in the noncompetitive binding assay on the membrane. Conversely, in the ELISA experiment, competition with the peptide epitope VVSHFND which is known to bind to the paratope[17] is monitored, meaning that only effects of the desired 1,3,5-triazine epitope

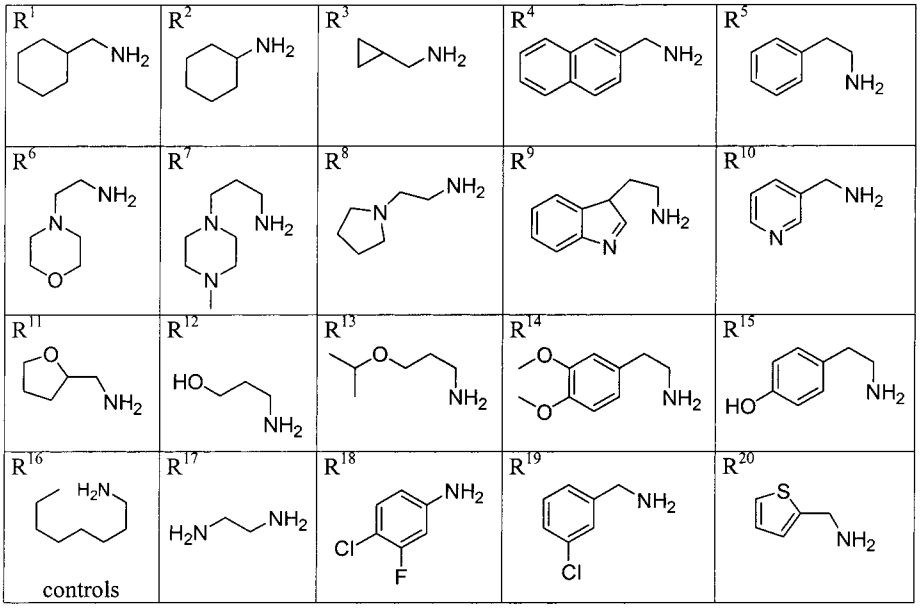


Figure 6. Employed amines R^1-R^{20} for the substitution of one of the chlorine atoms of the membrane-bound dichlorotriazines (control VVSHFND). The position in the table represents the position of the building block in the spatially addressed library (Figure 5).

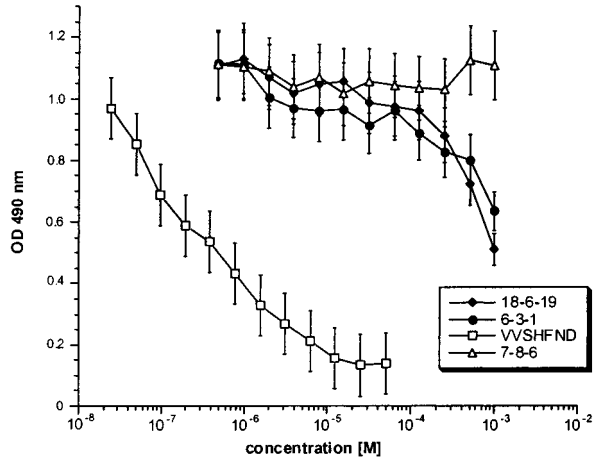


Figure 7. Inhibition of the mab Tab2 binding to the solid phase-adsorbed peptide epitope VVSHFNDCPDSHTQFAF by 1,3,5-triazines **18-6-19** and **6-3-1** and peptide VVSHFND (positive control) measured by ELISA. Compound **7-8-6** was included as negative control.

mimics binding to the paratope are detected. These effects have been described in detail for mab binding peptides derived from antigen protein sequences.[18] (2) Binding assays on cellulose membranes are enormously sensitive thus allowing for the detection of interactions with dissociation constants up to the millimolar range which cannot be measured in standard ELISA or plasmon surface resonance experiments.[19] It is assumed that this sensitivity is based on the high compound density which is calculated to be at least "millimolar" with regard to the volume of the SPOTs. (3) The conformational restriction of the dipeptide substituent by C-terminal bonding to the membrane enhances the binding properties of the 1,3,5-triazines relative to those of the soluble compounds. In addition, the 4,7,10-trioxa-1,13-tridecanediamine spacer to which only membrane-bound triazines are coupled could make additional contacts thereby increasing the affinity. Regardless of the fact that any one or a combination of these explanations is valid, compounds without inhibitory effects are ruled out by the competitive ELISA.

Conclusions

An effective synthetic protocol was developed, enabling spatially addressable SPOT-synthesis of large arrays of 1,3,5-triazines on planar surfaces and thereby extending the scope of the technology to the assembly of small heterocyclic compound libraries. Introduction of two different acid-labile linker systems and microwave-assisted nucleophilic substitution under mild conditions allowed the assembly of amino- and amino-oxy-1,3,5-triazines not previously readily accessible on solid support. In general, selective substitution of a single chlorine at membrane-bound dichloro-1,3,5-triazines could be achieved with amines having boiling points higher than 50 °C and solubilities of 1 M or better in NMP. Weak nucleophiles, such as 4-nitroaniline, or sterically hindered amines or alcohols resulted in the formation products of lower yields and purities at room and elevated temperatures. Addition of trimethylsilyl chloride (amines) or application of cesium salts (alcohols and phenols) resulted in increased substitution yields. Slow nucleophilic substitution at polymer-bound monochlorotriazines could be greatly facilitated under microwave irradiation, enabling the use of a wide variety of amines and phenols at cellulose and pp-membranes.

Cleavage of the generated compounds could be achieved under mild conditions by TFA vapor, leaving the compounds adsorbed on the polymeric support, thereby remaining spatially addressed. The synthetic conditions developed were applied to the highly parallel assembly of 8000 cellulose-bound 1,3,5-triazines which could be screened heterogeneously directly on the planar surface. Clearly, the method is of high potential for the parallel screening of membrane-bound small molecule compound libraries.

Experimental Section

Materials. Cellulose sheets (Whatman 50) were purchased from Whatman, U.K. Pp-membranes were from Millipore, Germany, and photocrafted by Poly-An GmbH, Germany. Reagents were obtained from Sigma-Aldrich GmbH, Germany, and Lancaster Synthesis GmbH, Germany, except bulk solvents which were purchased from Merck KGaA, Germany. Amino acid derivatives were provided by Calbiochem-Novabiochem GmbH, Germany.

Washing. All membranes were washed in a stainless steel dish between each reaction step (if not otherwise noted) with DMF, MeOH, and DCM three times each.

Cleavage. A desiccator was equipped with a glass dish containing 5 mL of TFA and evacuated to ~700 mbar for 1 h. The membranes containing compounds immobilized by the Rink or carbamate linker were placed in the TFA vapor filled desiccator for 30 min. Finally, the membranes were liberated from absorbed TFA in a vacuum for 120 min. Alternatively, single membrane disks (SPOTs) were punched out into 2 mL Eppendorf tubes. A total of 100 μL of 80% TFA in DCM was added, and the tube was shaken for 30 min. The solvent was removed in a gentle air stream.

Analysis. An HPLC-MS technique on an HP 1100 LC (Hewlett-Packard) combined with an LCQ-MS instrument (Finnigan MAT, Germany) equipped with an ESI source was used as a standard analytical method. All HPLC experiments were carried out on a VYDAC C-18 column (15 × 0.21 cm) using a linear gradient: eluent A 0.05% TFA in water; eluent B 0.05% TFA in acetonitrile, $\lambda = 220$ nm; flow rate 0.3 mL/min.

Amino-Functionalized Pp-Membrane (3). A carboxyl-functionalized pp-membrane **1** was immersed in a 2 M solution of PCl_5 in DCM for 2 h. The solution was removed and the membrane suffused with a 5 M solution of 4,7,10-trioxa-1,13-tridecanediamine in DCM for another 2 h. The membrane was washed with 0.5 M NaOH in water, then water alone, and finally by the standard washing procedure.

Alternatively, a methyl ester-modified pp-membrane **2** was immersed in 4,7,10-trioxa-1,13-tridecanediamine (neat) and warmed on a heatable shaker to 80 °C for 48 h followed by washing and drying in air. Amino functionalization was checked by pipetting 2 μL of a 0.6 M solution of Fmoc-β-Ala-OH/TBTU/NMI (1:1:1) (10 min preactivation time) in NMP directly onto the membranes. This procedure was repeated twice after 20 min, followed by washing, punch-pressing of single SPOTs, and Fmoc cleavage with 20% piperidine in DMF (30 min). The UV absorbance of the released dibenzofulvene/piperidine adduct was measured ($\epsilon_{301} = 7800$).

The results were confirmed by immersing amino-functionalized SPOTs in a 0.6 M solution of activated Fmoc-β-Ala-OH for 2 h in an Eppendorf tube to give identical results within the range of error (5%).

Rink Linker Functionalized Pp-Membrane (4/5). *p*-[(*R*,*S*)-α-[1-(9*H*-Fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid was preactivated with pentafluorophenol and *N*,*N*′-diisopropylcarbodiimide (DIC) (1:1:1) for 30 min in NMP (0.2 M). Then, 1 or 2 μL were pipetted onto the cellulose or pp-membrane, respectively, on a 1 × 1 cm grid, which was drawn on the cellulose or pp-membrane with a soft pencil prior to the pipetting steps. The procedure was repeated twice after 20 min followed by washing.

Aldehyde Membrane (6). Compound **3** was immersed in a 2 M solution of bromoacetyl bromide in DCM containing 0.3 M 1,4-diazabicyclo[2.2.2]octane (DABCO) for 30 min followed by the standard washing procedure. A total of 1 μL of an 1 M solution of the cesium salt of *p*-hydroxybenzaldehyde (formed from *p*-hydroxybenzaldehyde and Cs_2CO_3 2:1 in DMSO) was pipetted onto a membrane laid out in the form of a 1 × 1 cm grid. After 30 min the procedure was repeated twice. For removal of the unreacted bromo functions, the membrane was washed with a 5 M NaOMe solution in MeOH for another 45 min followed by the standard washing procedure to give **6**.

***p*-Nitrophenol Membrane (7).** Compound **6** was immersed in a 2 M solution of $NaBH_4$ in MeOH for 15 min, washed with MeOH twice, and then subjected to the standard washing procedure. The air-dried membrane was immersed in a 1 M solution of 4-nitrophenyl chloroformate in DCM containing a catalytic amount of NEM for 1 h followed by the standard washing procedure to give **7**.

Amino-Functionalized Membrane (7). A total of 2 μL of a 6 M solution of the diamine in NMP was spotted on a membrane laid out as a 1 × 1 cm grid. After 30, 60, and 90 min the procedure was repeated, followed by washing and drying in air.

Dichloro-1,3,5-triazine Membrane (8/9/10). Deprotected **4**/**5** (20% piperidine in DMF, 30 min) and amino-modified **7** were immersed in a 4 M solution of cyanuric chloride in methylene chloride (DCM) for 15 min giving **8/9/10**, respectively.

Monochloro-1,3,5-triazine Membrane (11/14). A total of 2 μL of a 3 M solution of the amine in NMP (2 μL of a 1 M solution of the cesium phenolate in DMSO) was spotted on a membrane laid out as a 1 × 1 cm grid. In the case of phenolate ions, the procedure was repeated after 15 min. After 30 min the membrane was washed and dried in air.

1,3,5-Triazine Membrane. A total of 2 μL of a 5 M solution of the amine in NMP (2 μL of a 1 M solution of the cesium-phenolate in DMSO) was spotted on a membrane laid out as a 1 × 1 cm grid. The membrane was heated by microwave irradiation (600 W) for 3 min, washed, and dried on air.

8000-Compound Library. Twenty replica of 400 dipeptides were synthesized via the SPOT technique[9] using an ABIMED ASP 222 robot (Abimed GmbH, Germany) on an 18 × 26 cm amino-functionalized cellulose membrane. After removal of the N-terminal Fmoc group (2 × 20 min, 20% piperidine in DMF (v/v)) and washing, the cellulose membrane was immersed in a 2 M solution of cyanuric chloride in DCM. The membrane was washed again and the 20 amines were spotted according to Figure 6. The amines (3 M solution in NMP) were allowed to react with the membrane-bound dichlorotriazines for 30 min, and the excesses were removed by the standard washing proccedure. The chlorines of the resulting membrane-bound monochlorotriazines were replaced with a 50% solution of piperidine in DMF (v/v) under microwave irradiation (2 × 3 min), and the excess piperidine was removed by the standard washing procedure.

Antibody Tab2 Binding Assay. The cellulose-bound library was washed once with methanol for 5 min and three times with TBS (Tris-buffered saline, pH 8.0) for 10 min each. The membrane was blocked for 2 h with 50 mL of blocking reagent (Boehringer Mannheim, Germany) in TBS and incubated with 1 μg/mL of monoclonal antibody Tab2 in blocking buffer for 2 h. After washing three times for 1 min with TBS/0.05% Tween 20 (T-TBS), 1,3,5-triazine-bound Tab2 antibody was detected by incubating the membrane with a second peroxidase-labeled anti-mouse IgG antibody (Sigma, Germany) at a concentration of 1 μg/mL in blocking buffer for 2 h. After washing three times for 5 min with T-TBS, enzymatic activity was measured and quantified using a chemoluminescence substrate (Boehringer Mannheim) on a LumiImager (Boehringer Mannheim) in Boehringer Light Units (BLU).

ELISA. Inhibitory effects of the 1,3,5-triazines were determined by competitive ELISA.[20] Microtiter plates (Maxisorp, 96 wells; Nunc, Denmark) were coated overnight with 50 μL of peptide VVSHFNDCPDSHTQFAF (1 μg/mL) in carbonate buffer (pH 9.6) at 4 °C. After washing three times with PBS (phosphate-buffered saline)/0.1% Tween 20, wells were incubated with different concentrations of 1,3,5-triazines and peroxidase-labeled[21] mab Tab2 (0.2 mg/mL) in PBS/0.1% Tween20 with 6% GelifundolS (Biotest Pharma, Germany) for 4 h at room temperature. After washing three times with PBS/0.1% Tween20, the bound enzymatic activity was measured by adding 5.5 mM *o*-phenylenediamine hydrochloride (Fluka, Switzerland) and 8.5 mM in 0.1 M citrate buffer pH 5.0, and was terminated after reaching optical densities around 1.0 (10 min) by adding 1 M sulfuric acid containing 0.05 M sodium sulfite. The absorbance was measured at 490 nm (ELISA-reader; Anthos, Germany). Inhibition constants (Table 4) were determined in duplicate and calculated by Klotz plotting 100%/(100% OD) versus $1/c$ (100% = OD mean value without any competitor; c = inhibitor concentration) as described.[20]

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Supporting Information Available. Purities of crude 1,3,5-triazines, obtained by selective "spot-wise" nucleophilic substitution of a single chlorine at cellulose or PP-membrane-bound dichlorotriazines, subsequent replacement of remaining chlorines by an appropriate amine,[14] and cleavage by TFA. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) For a complete list of applied building blocks, refer to the Supporting Information.
(14) Conversion was studied as follows: (i) Pipetting of 2−5 M solutions (depending on the solubility) of amines onto dichlorotriazines immobilized with the Rink linker on pp-membrane and cellulose was followed by washing of the membranes after 30 min. (ii) To use UV quantification of purities and conversions, all remaining chlorines (nonreacted chlorines of dichlorotriazines and chlorines of monochlorotriazines) were quenched with either benzylamine (when aromatic amines were tested in step i) or piperidine (when aliphatic amines were tested in step i) to give compounds with similar extinction coefficients. Membranes were immersed in the corresponding amine solution and exposed to microwave irradiation for 2 × 3 min to ensure complete substitution. (iii) After washing and drying of the membranes, compounds were cleaved from the membranes with TFA vapor, and conversion and purity were analyzed by LC-MS.
(15) Several aniline substituted triazines were synthesized on Rink linker modified cellulose and pp-membranes under comparable conditions. Analysis of the cleaved products revealed average purities of 80% from the pp-membrane case compared to 50% purity from cellulose.
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